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Characterisation of proanthocyanidin aglycones and glycosides from rose hips by high-performance liquid chromatography–mass spectrometry, and their rapid quantification together with Vitamin C

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Abstract

Fifteen individual proanthocyanidin aglycones and 19 glycosides, together with a complex mixture of chromatographically non-separated tetra- to octameric proanthocyanidin glycosides were detected—the non-separated glycosides being novel natural products—and characterised from dog rose hips using high-performance liquid chromatography–electrospray ionisation mass spectrometry (HPLC–ESI-MS). Along with these phenolics, a 50% aqueous ethanol extract of rose hips was found to contain high levels of Vitamin C. A simple and rapid HPLC method assisted by diode array detection for the estimation of the total concentration of proanthocyanidin aglycones and glycosides, as well as Vitamin C, in rose hip extracts was developed.

Keywords: Rose hips; Proanthocyanidins; Proanthocyanidin glycosides; Vitamin C; HPLC; ESI-MS; Rapid quantitative analysis

1. Introduction

There is an increasing global interest towards developing so-called functional foods or finding food additives that would be able to protect human body from diseases caused by, e.g., oxidative stress in human cells. A large number of plant derived antioxidant compounds have already been identified, and the most important ones are Vitamin C, Vitamin E, several phenolic compounds and carotenoids [1–3]. However, a single compound or group of compounds may not restrict the oxidative damage in a sufficient extent. Rather, a number of dietary compounds with variable antioxidative effects guarantee the best prevention against an antioxidative related disease [2]. In addition to the known antioxidants, and to widen the scope of their effects, we would need to find a group of compounds that would be novel in its structure, highly abundant in a plant part or plant species, and possess a high antioxidant activity.

Fruits of roses (*Rosa* L.), i.e., rose hips, are a multiple source of antioxidants, as they are rich in vitamins C and E, carotenoids and phenolic compounds such as flavonoid glycosides and proanthocyanidin aglycones [4–8]. Maybe for these reasons rose hips have been used, e.g., as a food source [9] and as medicine in ethnopharmacology [4,10]. In our preliminary high-performance liquid chromatographic (HPLC) analyses of hips of three dog rose species, i.e., *Rosa dumalis*, *R. mollis* and *R. sherardii*, we found high levels of unusual, very rarely reported, and even novel proanthocyanidin glycosides in all three species. Therefore, in this paper, we report the characterisation of these glycosides and also aglycones of proanthocyanidins (PAs, condensed tannins), from dog rose hip extracts using HPLC connected to electrospray ionisation mass spectrometer (ESI-MS). Also a simple and rapid

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method using HPLC with a diode array detector (DAD) for the estimation of the content of these rare tannins, as well as Vitamin C, in rose hip extracts is presented.

2. Experimental

2.1. Sample preparation

Hips of three dog rose species (*R. dumalis* Bechst., *R. mollis* Sm. and *R. sherardii* Davies) were sampled in the Botanical garden of the University of Turku, SW-Finland, during August and September of both 2002 and 2003. There were 25 *R. dumalis*, 11 *R. mollis*, and four *R. sherardii* bushes. Each bush was sampled at least twice during the sampling period. Seeds were removed from the collected plant material that was further freeze-dried and homogenised into fine powder.

In the extraction solvent comparisons, fine powder of rose hips (200 mg dry mass) was extracted with 10 mL of one of the following solvents: 50%, 70% and 85% aq. Me_2CO, and 100% Me_2CO, and 50%, 70% and 85% aq. EtOH, and 100% EtOH. After centrifuging at 2000 × *g* for 10 min, the insoluble residue was re-extracted twice with the same solvent. Me_2CO or EtOH was evaporated from the combined extract under reduced pressure at 40 °C. The water-soluble residue was freeze-dried and dissolved in 10 mL water. Samples were filtered through 0.45 μm PTFE filters before HPLC–DAD analysis.

In the final extraction method, 50% aq. EtOH (adjusted to pH 2 with 0.05 M H_3PO_4 to stabilise Vitamin C) was chosen as the best extraction solvent for both PA glycosides, and Vitamin C. Sample amount was adjusted to 10 mg that was extracted twice (2 × 1 h) with 800 μL of 50% aq. EtOH (pH 2). The third extraction was shown to extract no additional analytes from the insoluble residue, but it cannot be claimed that the two extractions were 100% quantitative. Traces of the analytes could have been still left non-extracted, especially since plant PAs are often found also in insoluble, cell-wall bound forms; these would require, e.g., acid-hydrolysis in order to be released from the plant material. Finally, the combined 1.6 mL extract was filtered through a 0.45 μm PTFE filter and analysed directly with HPLC–DAD, HPLC–ESI-MS, or the HCl–butanol assay, without any other sample preparation or purification steps that could have caused recovery losses of the analytes.

In the preparative-scale extractions fine powder of rose hips (200 g dry mass; samples from all three rose species had to be combined to gather such a large amount) was extracted with 50% aq. EtOH (5 × 1 L). EtOH was evaporated from the combined extract by rotary evaporation at 40 °C. One litre of 100% Me_2CO was added to the water extract. The resulting orange, gum-like precipitate was separated from the supernatant that contained the PA glycosides. Me_2CO was further evaporated from the supernatant by rotary evaporation at 40 °C. The resulting water extract (50 mL) was applied into Sephadex LH-20 column (40 cm × 2.5 cm i.d., Pharmacia, Umeå, Sweden) and fractionated by consecutive elution with water, 10–100% aq. EtOH (in 10% intervals), and with 30%, 50% and 70% aq. Me_2CO. The 20% aq. EtOH fraction that contained high level of a PA dimer monoglycoside was purified further with the Sephadex LH-20 column using less steep gradient of 10–20% aq. EtOH. Final purification of the PA dimer monoglycoside was done with semi-preparative HPLC.

2.2. Standards and solvents

Me_2CO, MeCN, MeOH and BuOH were from Lab-Scan (Dublin, Ireland); HCl, HCOOH and H_3PO_4 were from J.T. Baker (Deventer, Holland). EtOH was from Altia (Rajamäki, Finland). Purified water was delivered by an Elgastat UHQ-PS purification system (Elga, Kaarst, Germany).

2.3. Equipment

2.3.1. HPLC–DAD analysis

Analytical chromatographic analysis was performed with an HPLC system (Merck-Hitachi, Tokyo, Japan) that consisted of a vacuum degasser L-7614, a pump L-7100, a programmable autosampler L-7250, a diode array detector L-7455 and an interface D-7000. The column was Superspher 100 RP-18 (75 mm × 4 mm i.d., 4 μm, Merck, Germany).

Semi-preparative HPLC was performed using a Merck-Hitachi L-6200A pump (Hitachi, Tokyo, Japan) and a Perkin-Elmer LC-235 diode array detector (Perkin-Elmer, Norwalk, CT, USA) connected to a Graphic Printer GP-100 (Perkin-Elmer, Beaconsfield, England). Injections were made via a Rheodyne rotary switching valve (Cotati, CA, USA) with a 500-μL loop. The column was LiChrospher 100 RP-18 (250 mm × 10 mm i.d., 10 μm, Merck, Germany).

2.3.2. HPLC–ESI-MS analysis

HPLC–ESI-MS analysis was performed using a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray interface. The HPLC system configuration and the columns used were the same as described earlier [11,12]. The mass spectrometer was operated both in the negative and positive modes. Mass spectra were obtained by acquiring data between 200 and 1600 or 2000 amu.

2.3.3. NMR analysis

NMR spectra were acquired using a Bruker Avance 500 spectrometer (equipped with BBO-5 mm-Zgrad probe) operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. Spectra were recorded at 25 °C using acetone-d_6 + D_2O as a solvent (D_2O for the better solubility of the sample) with a non-spinning sample in a 5 mm NMR tube. Spectra were processed by a PC with Windows XP operating system and XWin-NMR software. Proton and carbon spectra were referenced internally to TMS signal using value 0.00 ppm. In

addition to normal proton and carbon spectra, also two-dimensional DQF-COSY, HSQC and HMBC were recorded. All spectra were measured using standard pulse programs installed by Bruker.

2.3.4. HCl–butanol assays

The total content of PAs was measured with the HCl–butanol assay as in [13] using a Perkin-Elmer Lambda 12 UV–vis spectrometer (Norwalk, CT, USA).

2.4. Chromatographic conditions

2.4.1. Analytical HPLC–DAD analysis

Two solvents were used: (A) 0.05 M H_3PO_4; (B) MeCN. At first the elution profile was as described earlier [12]. The elution profile of the further developed HPLC program was: 0–2 min, 100% A (isocratic); 2–3.5 min, 0–70% B in A (linear gradient); 3.5–6 min, 70% B (isocratic); 6–6.5 min, 70–95% B in A (linear gradient); 6.5–8.5 min, 95% B (isocratic); 8.5–9.5 min, 95–0% B in A (linear gradient); 9.5–15 min, 100% A (isocratic); detection at 240 nm. Flow rate profile was: 0–6 min, 1 mL min^{-1}; 6–6.5 min, 1–2 mL min^{-1} (linear gradient); 6.5–13.5 min, 2 mL min^{-1}; 13.5–14 min, 2–1 mL min^{-1} (linear gradient); 14–15 min, 1 mL min^{-1}. The acquisition of UV spectra (195–700 nm) was done automatically at the apex of each peak.

2.4.2. Semi-preparative HPLC analysis

Two solvents were used: (A) 1.0 % HCOOH; (B) MeCN. The elution profile was: 0–5 min, 100% A (isocratic); 5–60 min, 0–30% B in A (linear gradient); 60–70 min, 30–70% B in A (linear gradient). Flow rate, 6 mL min^{-1}; detection at 280 nm.

2.4.3. HPLC–ESI-MS analysis

Reversed-phase HPLC–ESI-MS analysis was performed as by [12], except that nebuliser gas was set at position 9 and curtain gas at position 12. Normal-phase HPLC–ESI-MS analysis was performed as previously described in [11].

3. Results and discussion

3.1. Characterisation of the rose hip compounds from the crude extract

Rose hips are known to contain high levels of Vitamin C, which has a UV maximum at around 240 nm; therefore 240 nm was used as the main acquisition wavelength in the HPLC–DAD analyses. Indeed, each of the three rose species studied were found to contain Vitamin C in their hips (the peak at around 1 min in Fig. 1). Otherwise the HPLC traces of rose hips were surprisingly complex; a chromatographically non-separated hump occurred at the 10–22 min area (Fig. 1). Interestingly, the 3-D contour graph of the HPLC–DAD run revealed that the whole hump had a UV spectrum similar to that of a flavan-3-ol (e.g., catechin), the monomeric building block of PA aglycones. Moreover, similar humps occur with plant samples containing high levels of oligo- (higher than trimers) or polymeric PA aglycones; these types of compounds cannot be separated by reversed-phase HPLC but need normal-phase HPLC instead [11]. Thus, to achieve a

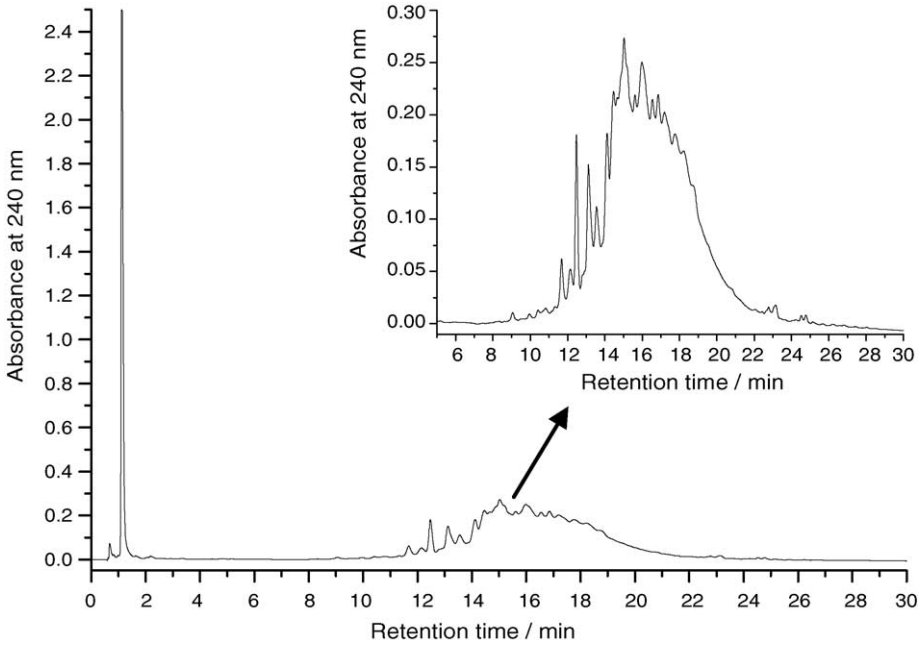


Fig. 1. HPLC traces of a 50% aq. ethanolic dog rose hip extract (made from a combined hip sample of three dog rose species, *R. dumalis*, *R. mollis* and *R. sherardii*) run with a Merck Superspher 100 RP-18 column (75 mm × 4 mm i.d., 3 μm) and a gradient consisting of 0.05 M H_3PO_4 (A) and MeCN (B): 0–3 min, 100% A (isocratic); 3–30 min, 0–30% B in A (linear gradient); flow rate 1 mL min^{-1}.

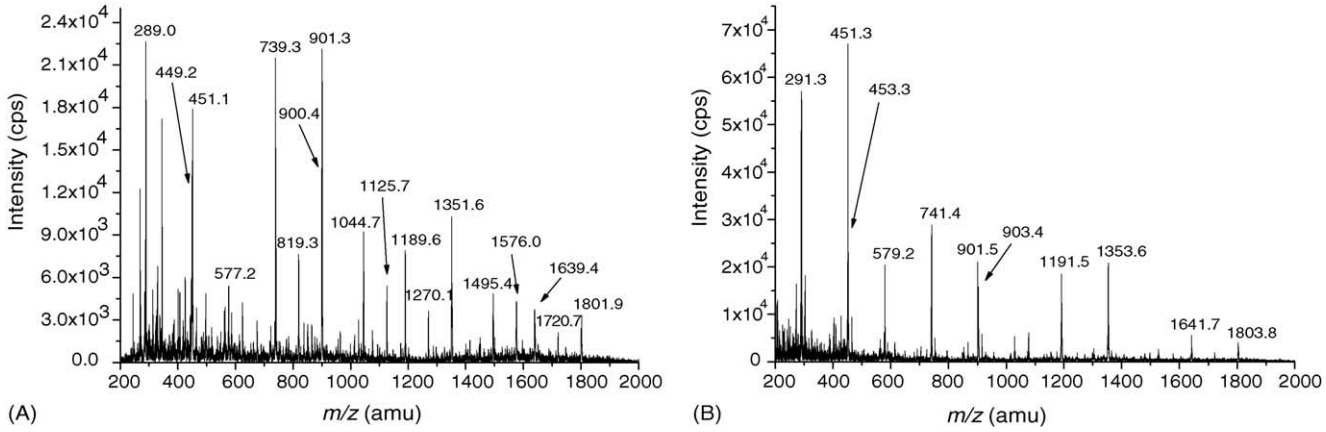


Fig. 2. Negative (A) and positive (B) ion mass spectra recorded from the proanthocyanidin part of an HPLC–ESI-MS run of a crude rose hip extract made from a combined sample of three dog rose species, *R. dumalis*, *R. mollis* and *R. sherardii*. Preliminary identification of the main tannins on the basis of negative ions: $[M-H]^-$ of the monomer aglycone at *m/z* 289.0; $[M-H]^-$ of the monomer glycoside at *m/z* 451.1; $[M-H]^-$ of the dimer aglycone at *m/z* 577.2; $[M-H]^-$ of the dimer glycoside at *m/z* 739.3; $[M-2H]^{2-}$ of the tetramer triglycoside at *m/z* 819.3; $[M-2H]^{2-}$ of the tetramer tetraglycoside at *m/z* 900.4; $[M-H]^-$ of the dimer diglycoside at *m/z* 901.3; $[M-2H]^{2-}$ of the pentamer tetraglycoside at *m/z* 1044.7; $[M-2H]^{2-}$ of the pentamer pentaglycoside at *m/z* 1125.7; $[M-H]^-$ of the trimer diglycoside at *m/z* 1189.6. The *m/z* values at 1270.1, 1351.6, 1495.4, 1576.0, 1639.4, 1720.7 and 1801.9 would match with the $[M-2H]^{2-}$ ions of a hexamer pentaglycoside, a hexamer hexaglycoside, a heptamer hexaglycoside, a heptamer heptaglycoside, an octamer hexaglycoside, an octamer heptaglycoside and an octamer octaglycoside, respectively.

better chromatographic separation with the hump compounds, we ran a few extracts with normal-phase HPLC as well. However, the normal-phase analysis of rose hips revealed a poorer separation of peaks than with other plant extracts studied with the same method (data not shown). Moreover, instead of a clear peak that would have been characteristic to polymeric PA aglycones [11], an unusual large hump was detected at the end of the chromatogram. Therefore, we concluded that the rose hip PAs must have some unusual structural characteristics which would explain their peculiar chromatographic performance in comparison to the PA aglycones commonly found in other plants. Indeed, this was confirmed by additional reversed-phase analyses; negative and positive ion electrospray ionisation mass spectrometric runs of the extracts (see Fig. 2) showed only a few *m/z* values corresponding to the commonly known PA aglycones (*m/z* values at 289.0 and 291.3, and at 577.2 and 579.2 corresponded to $[M-H]^-$ and $[M+H]^+$ of PA aglycone monomers and dimers, respectively). Surprisingly, instead of referring to PA aglycones, the rest of the *m/z* values shown in Fig. 2 matched with PA mono- to octamers having one to eight additional sugar units attached to them. For instance, *m/z* 739.3 ($[M-H]^-$) in Fig. 2A and *m/z* 741.4 ($[M+H]^+$) in Fig. 2B matched with a PA dimer monoglycoside, and *m/z* values at 1801.9 ($[M-2H]^{2-}$) and at 1803.8 ($[M+2H]^{2+}$) with a PA octamer octaglycoside (for more examples see the legend of Fig. 2). Mass spectra of Fig. 2 were run from a crude rose hip extract made from a combined sample of three rose species, i.e., *R. dumalis*, *R. mollis* and *R. sherardii*. When the crude extracts of each species were analysed separately with negative ion HPLC–ESI-MS, the *m/z* values of PA glycosides were shown to dominate the mass spectra just like in Fig. 2A. In our previous studies with, e.g., pine bark and leaves of deciduous trees ([11], Karonen and Salminen, unpublished data), we had always detected various PA aglycones from the extracts, but this was the first time that we found PA glycosides. Actually, to the best of our knowledge, PA glycosides larger than trimers were now found for the first time in Nature on the whole.

3.2. Characterisation of proanthocyanidins from the Sephadex LH-20 fractions

To achieve a more detailed characterisation of the preliminarily identified PA aglycones and especially the rare glycosides, the fractionation and purification of the crude extract was carried out. Plant extracts are traditionally fractionated by column chromatography with Sephadex LH-20. We

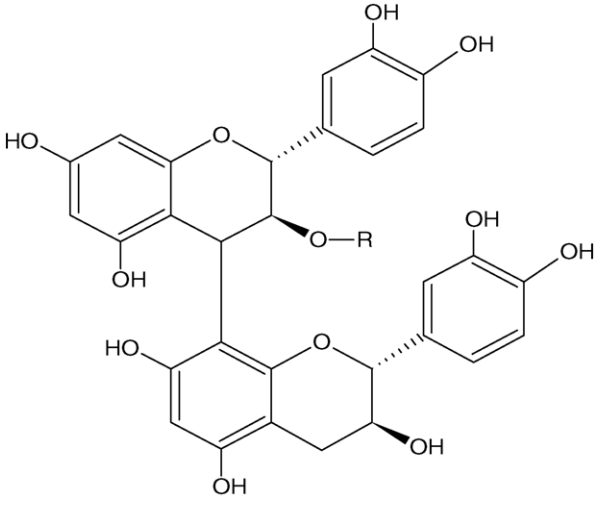


Fig. 3. Structures of a proanthocyanidin dimer aglycone (R = H) and a proanthocyanidin dimer glycoside (R = glucose).

Table 1
Individual proanthocyanidin aglycones and glycosides determined by negative ion HPLC–ESI-MS in the Sephadex LH-20 fractions of a dog rose hip extract

Tentative identification	R_t/min	$[M-H]^-$	$[2M-H]^-$	Fragments	Sephadex LH-20 fraction
Monomer aglycone	13.9	289.0	579.1	–	40% EtOH
Monomer glycoside	12.4	451.2	903.1	289.2	30% EtOH
Monomer glycoside	14.1	451.2	903.1	289.2	20% EtOH
Dimer aglycone	13.1	577.2	1155.5	289.0, 407.2, 425.2, 451.1	40% EtOH
Dimer aglycone	13.3	577.2	1155.4	289.0, 407.2, 425.3, 450.9	40% EtOH
Dimer aglycone	16.5	577.2	1155.5	289.0, 407.2, 425.2, 451.1	60% EtOH
Dimer aglycone	18.4	577.2	1155.2	289.2, 407.1, 425.3, 451.0	60% EtOH
Dimer monoglycoside	10.5	739.3	1479.7	288.9, 406.9, 425.1, 449.2	20% EtOH
Dimer monoglycoside	12.7	739.3	1479.7	288.9, 406.9, 425.1, 449.2	30% EtOH
Dimer monoglycoside	14.5	739.3	1479.7	288.9, 406.9, 425.1, 449.2	40% EtOH
Dimer diglycoside	10.9	901.3	–	–	10% EtOH
Dimer diglycoside	13.0	901.3	–	–	20% EtOH
Trimer aglycone	10.1	865.4	–	575.1	40% EtOH
Trimer aglycone	14.2	865.3	–	288.9, 575.1	60% EtOH
Trimer aglycone	15.7	865.4	–	289.0, 739.2, 575.3	60% EtOH
Trimer aglycone	16.4	865.3	–	288.8	60% EtOH
Trimer aglycone	17.7	865.5	–	288.7, 713.1, 575.2	60% EtOH
Trimer aglycone	19.1	865.4	–	288.8	60% EtOH
Trimer monoglycoside	9.3	1027.6	–	–	20% EtOH
Trimer monoglycoside	11.8	1027.5	–	–	30% EtOH
Trimer monoglycoside	13.5	1027.5	–	–	40% EtOH
Trimer monoglycoside	15.5	1027.6	–	–	50% EtOH
Trimer monoglycoside	15.9	1027.5	–	–	50% EtOH
Trimer monoglycoside	16.8	1027.5	–	–	50% EtOH
Trimer diglycoside	8.9	1189.6	–	–	10% EtOH
Trimer diglycoside	10.3	1189.6	–	–	10% EtOH
Trimer diglycoside	11.9	1189.6	–	–	10% EtOH
Trimer diglycoside	12.1	1189.6	–	–	10% EtOH
Trimer diglycoside	13.6	1189.6	–	–	30% EtOH
Trimer diglycoside	14.5	1189.6	–	289.2, 739.2	30% EtOH
Tetramer aglycone	13.5	1153.5	–	–	70% EtOH
Tetramer aglycone	15.0	1153.6	–	288.7, 577.2, 865.5	70% EtOH
Tetramer aglycone	15.4	1153.6	–	–	70% EtOH
Tetramer aglycone	17.2	1153.5	–	288.7, 577.2, 865.3	70% EtOH

Compounds are listed in the order of increasing degree of polymerisation.

Table 2
Other phenolics than proanthocyanidins determined by negative ion HPLC–ESI-MS in the Sephadex LH-20 fractions of a dog rose hip extract

Tentative identification	R_t/min	$[M-H]^-$	$[2M-H]^-$	$[M-2H]^{2-}$	Fragments	Sephadex LH-20 fraction
Apigenin derivative[a]	19.2	449.3	899.5	–	269.0	20% EtOH
Eriodictyol hexoside[a]	20.2	449.2	899.1	–	287.1	30% EtOH
Methyl gallate rutinoside[a]	25.7	491.1	983.2	–	345.3, 182.9	30% EtOH
Pedunculagin[b]	9.6 and 12.5[c]	783.0	1567.2	391.2	301.0	50% EtOH
Pentagalloylglucose[b]	22.8	939.3	–	469.2	169.0, 393.1, 769.2	70% EtOH
Phloridzin[a]	24.2	434.9	871.1	–	272.9	40% EtOH
Quercetin hexuronide[a]	22.8	433.2	867.2	–	301.2	40% EtOH
Quercetin hexuronide[a]	21.7	477.0	955.0	–	300.7, 178.8	10% EtOH
Quercetin rhamnoside[a]	23.2	447.0	895.2	–	300.9	40% EtOH
Quercetin-3-galactoside[a]	21.2	463.2	927.3	–	301.0	40% EtOH
Rutin[a]	21.2	609.3	1219.4	–	300.9	30% EtOH
Taxifolin pentoside[a]	18.7	435.2	871.4	–	285.0, 302.8	30% EtOH
Taxifolin pentoside[a]	20.8	435.0	871.5	–	285.0, 302.9	30% EtOH
Tellimagrandin I[b]	14.1 and 16.8[c]	785.1	1571.2	–	301.1	60% EtOH

Compounds are listed in the alphabetical order.
[a] Tentatively identified by comparing *m*/*z* values and retention times to those reported by Hvattum [8].
[b] Tentatively identified by comparing *m*/*z* values and retention times to those reported by Salminen et al. [12,15].
[c] Mixture of α- and β-glucose isomers.

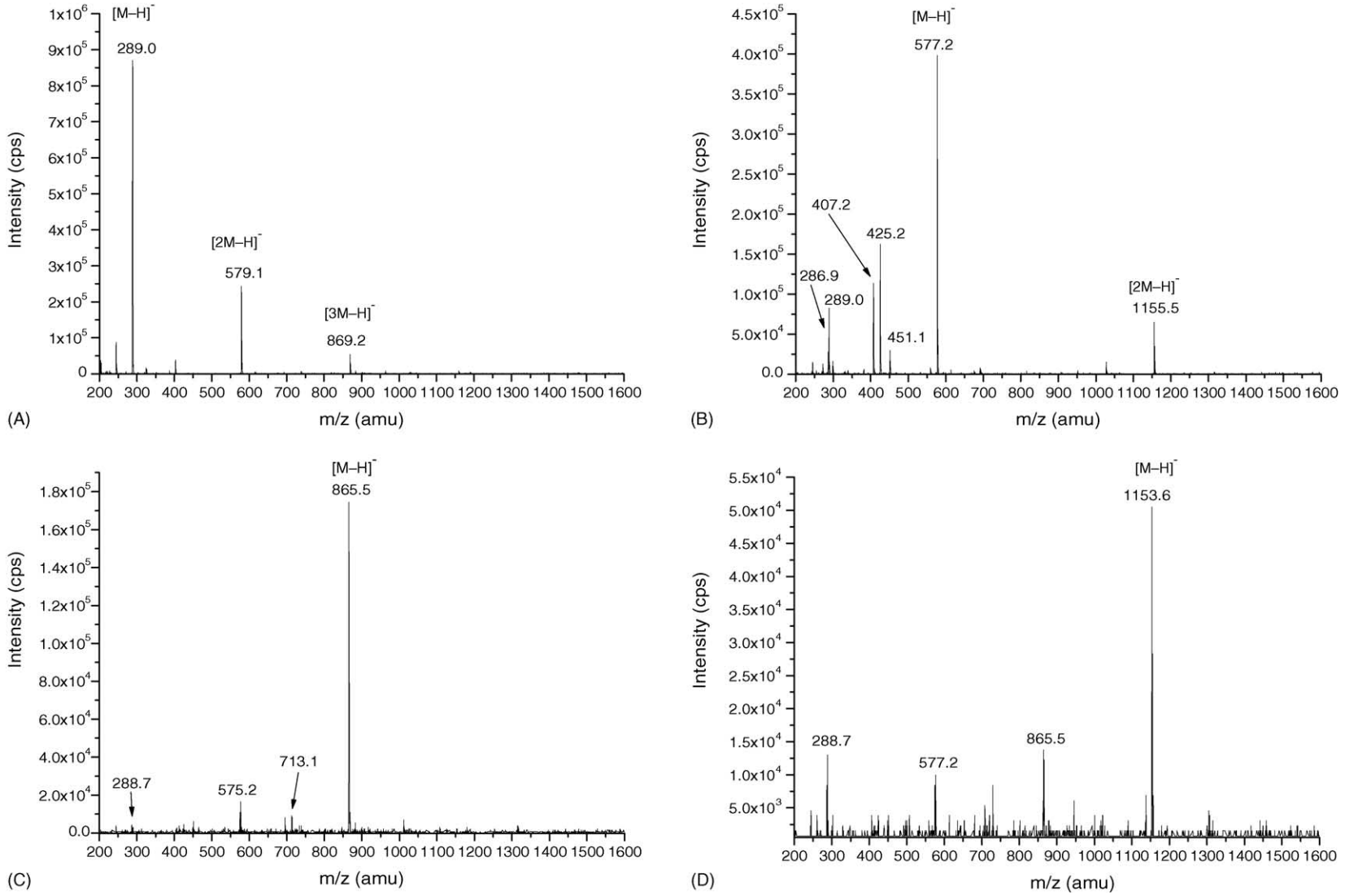


Fig. 4. Negative ion mass spectra of four proanthocyanidin (PA) aglycones (A–D) and those of four PA glycosides (E–H). (A): PA monomer aglycone, (B): PA dimer aglycone, (C): PA trimer aglycone, (D): PA tetramer aglycone, (E): PA monomer monoglycoside, (F): PA dimer monoglycoside, (G): PA trimer diglycoside, (H): PA tetramer triglycoside.

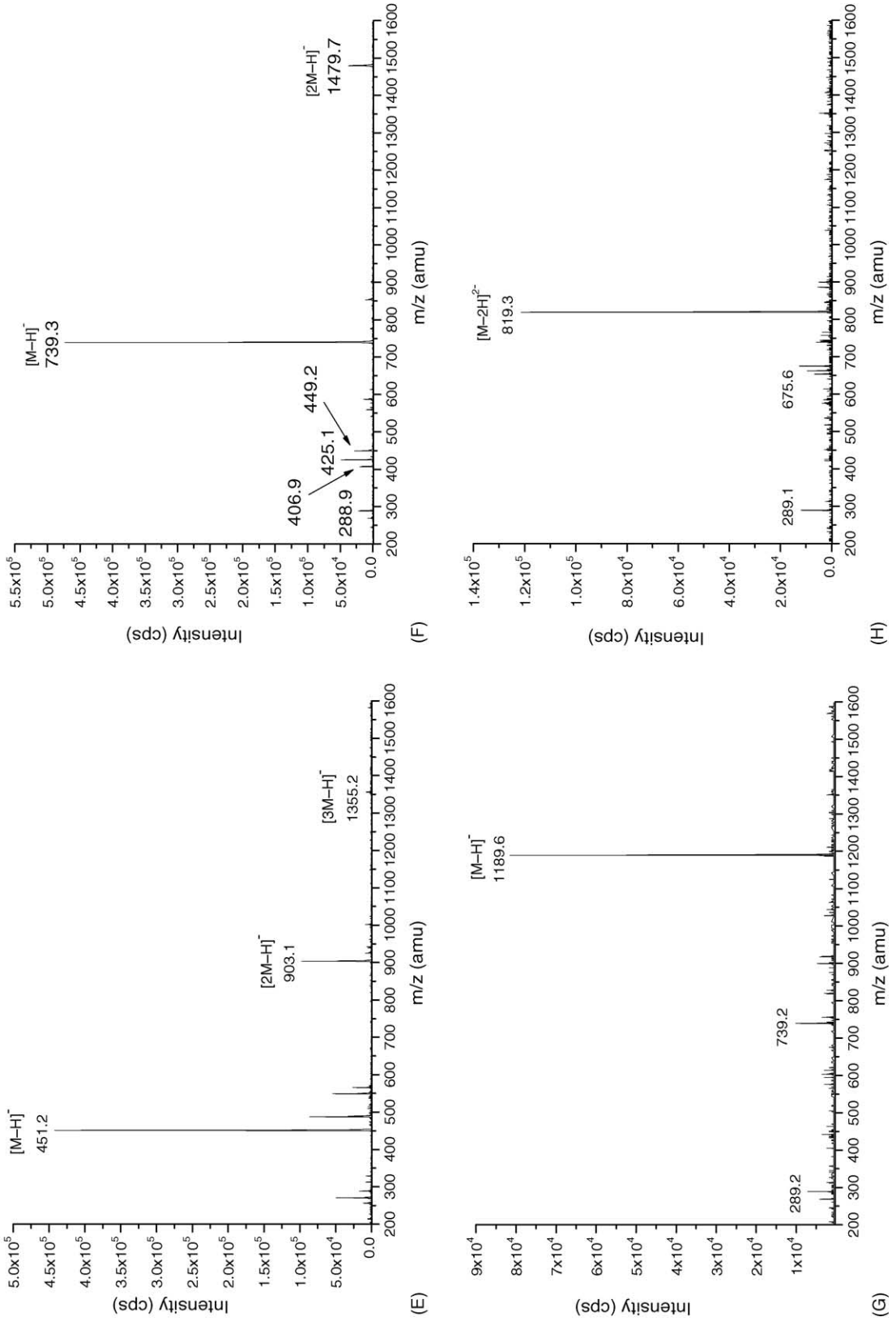


Fig. 4. (*Continued*).

performed the fractionation by consecutive elution with water, 10–100% aq. EtOH (in 10% intervals), and 30%, 50% and 70% aq. Me_2CO. This type of elution profile is known to yield mono- to tetrameric PA aglycones with 30–50% aq. EtOH and the polymeric ones with 50–70% aq. Me_2CO [11]. It is also known that flavonoid glycosides elute in Sephadex LH-20 fractionations before the corresponding aglycones (Salminen, unpublished observation). Therefore, it was not surprising that rose hip PA glycosides eluted already with 10–30% aq. EtOH, and the mono- to tetrameric aglycones just with 40–70% aq. EtOH; this rather confirmed the presence of sugar moieties in many of the rose hip PAs (Fig. 2). To prove this without any controversy, we isolated a PA dimer monoglycoside from the 20% aq. EtOH fraction with semi-preparative HPLC and subjected it to scrutinised ^1H and ^{13}C NMR analyses. Indeed, all the NMR signals (proton and carbon chemical shifts and proton–proton coupling constants), as well as the rotameric behaviour of the structure in acetone/D_2O mixture, were found to match with those reported by [14] for a monoglucoside of a catechin–catechin type dimer (Fig. 3).

The negative ion HPLC–ESI-MS runs of all the fractions revealed the presence of 15 individual PA aglycones and 19 PA glycosides in rose hips (Table 1). In the cases of shorter chain PAs the main peak in the mass spectra of an individual tannin was the deprotonated molecule $[M-H]^-$. However, when the molar mass of the compound exceeded approximately 1600 g/mol, the doubly charged deprotonated molecule $[M-2H]^{2-}$ was more dominant than $[M-H]^-$; a similar pattern observed for PA aglycones by [11]. Therefore, to increase the sensitivity of the ESI-MS analysis, the acquired atomic mass unit area of the purified fractions was restricted to 200–1600 amu. Fig. 4 shows examples of mass spectra obtained from the purified fractions for four aglycones and the corresponding glycosides. Of especial interest were the mass spectral fragmentations of the PA dimer aglycone and its monoglucoside (Fig. 4B and F). The fragmentation mechanisms (retro-Diels-Alder (RDA), quinone methide (QM) cleavage and heterocyclic ring fission (HRF)) of a dimer aglycone in ESI-MS are dealt in detail by [11]. Here, for the very first time, similar fragmentations were found to occur in ESI-MS with dimer glycosides as well. The RDA fragmentation produced the ion at *m/z* 425.1, i.e., $[M–C_{14}H_{18}O_8–H]^-$ while the *m/z* 406.9 was the ion formed from the elimination of water from *m/z* 425.1 (Fig. 4F). For the dimer glycoside, QM cleavage was shown to produce ions at *m/z* 449.2 (corresponding to the upper, i.e., extension unit) and at 288.9 (corresponding to the lower, i.e., terminal unit); this further confirmed that the glucose moiety was attached to the extension unit of the glycoside. The presence of HRF in ESI-MS of dimer glycosides was less evident; only traces of the corresponding ion at *m/z* 613.2 (i.e., $[M–C_6H_6O_3–H]^-$) were found and it is presumable that the glucose at C-3 inhibited HRF of the dimer glycoside. Actually, also RDA and QM fragmentation mechanisms were less efficient with the dimer glycoside than with the corresponding aglycone (compare the proportional intensities of the fragments to the intensities of the deprotonated molecules at *m/z* 577.2 and at *m/z* 739.3 in Fig. 4B and F).

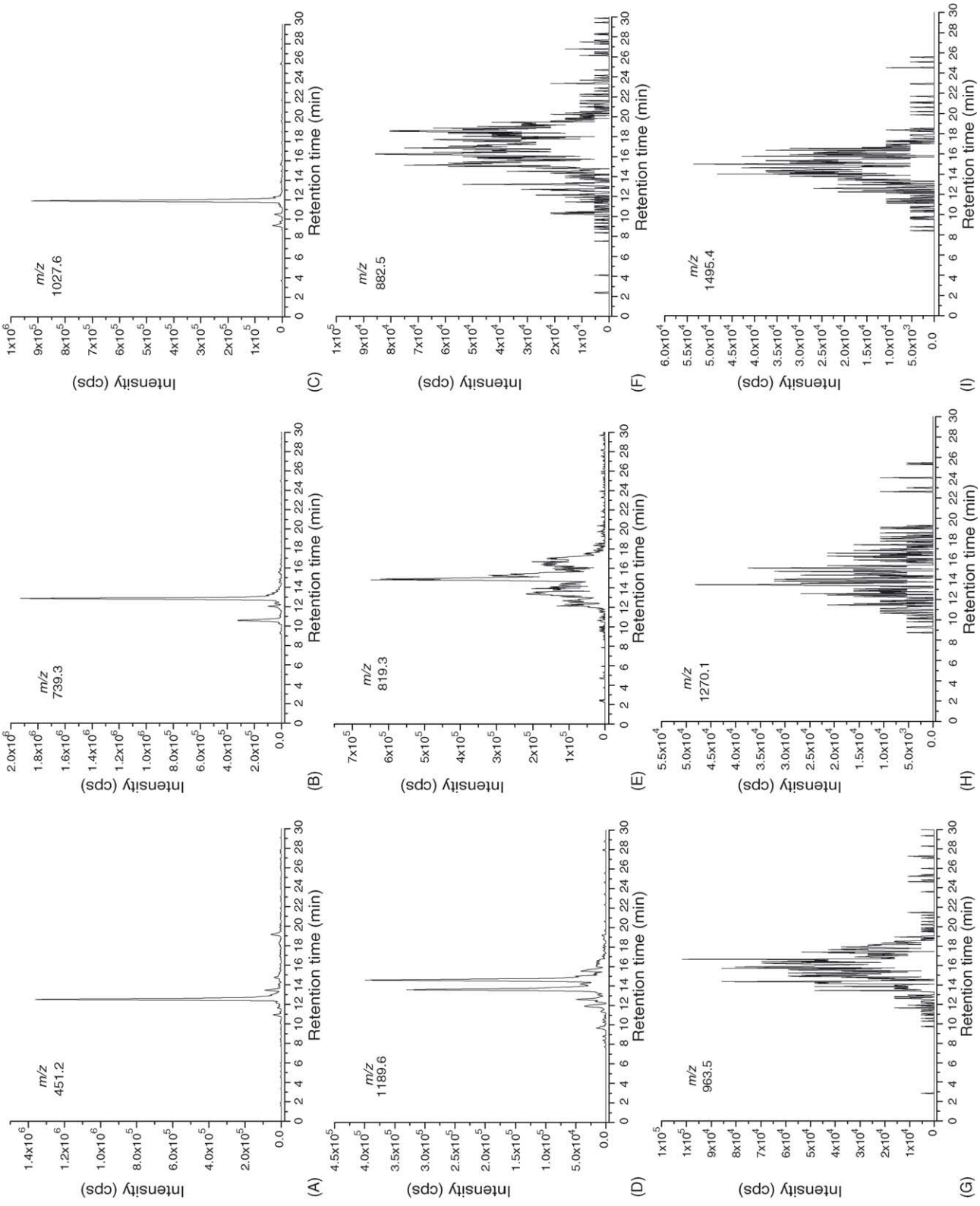
The LC–MS analyses of the fractions revealed that the chromatographic hump of Fig. 1 was mainly due to PA oligomer glycosides; the hump was found in the 10–40% aq. EtOH fractions and not in the Me_2CO fractions that are typical to PA aglycone polymers [11]. Fig. 5 emphasises that PA glycosides up to trimers were separated as single peaks (Fig. 5A–D), but from tetramers onwards (Fig. 5E–I) the separation got worse and resulted in a hump. At the same time the ionisation seemed to deteriorate as the degree of polymerisation of the glycosides increased; a similar pattern has been observed for PA aglycones by [11]. The co-elution of tetra- to octameric PA glycosides under the hump prevented the calculation of the absolute numbers of individual PA glycosides present in dog rose hips. Nevertheless, it is clear that dog rose hips contain an exceptionally complex mixture of PA glycosides, the largest individual compound found in this study being a PA octamer octaglycoside. With such a rare composition of these glycosides, some of which are even novel natural products, dog roses and their hips are distinctive from most other plant species, or plant parts.

3.3. Characterisation of other phenolics than proanthocyanidins from the Sephadex LH-20 fractions

Although the HPLC–DAD analysis evidenced the presence of mainly PA aglycones and glycosides together with Vitamin C in a crude dog rose hip extract, the Sephadex LH-20 fractionations uncovered traces of additional phenolics in hips as well (see Table 2). Eleven of these compounds were tentatively identified by direct comparisons with the retention times and *m/z* values reported by [8] for phenolics found in a *R. canina* hip extract. Three hydrolysable tannins, i.e., pedunculagin, tellimagrandin I and pentagalloylglucose, were tentatively identified for the first time from dog rose hips by matching chromatographic properties with those earlier reported by us from birch (*Betula pubescens*) leaves [12,15].

3.4. Quantification of proanthocyanidin aglycones and glycosides from a crude rose hip extract

Already Fig. 1 showed that only a few rose hip PA aglycones or glycosides could be quantified individually from a crude rose hip extract with reversed-phase HPLC–DAD; mainly an indefinite hump was detected instead of nicely separated peaks. In principal, the problem of PA co-elution could have been overcome by selected ion monitoring of, e.g., deprotonated molecules of individual PAs with HPLC–ESI-MS. This approach, however, was not feasible due to poor ionisation of PAs having degree of polymerisation higher than three (Fig. 5). At its worst, this would have resulted into severe underestimations of PA contents of rose hips, together with non-reproducible quantifications caused by inadequate efficiency in the ionisation of high-molecular-weight


(A)
m/z
451.2
(B)
m/z
739.3
(C)
m/z
1027.6
(D)
m/z
1189.6
(E)
m/z
819.3
(F)
m/z
882.5
(G)
m/z
963.5
(H)
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1270.1
(I)
m/z
1495.4
Intensity (cps)
Retention time (min)

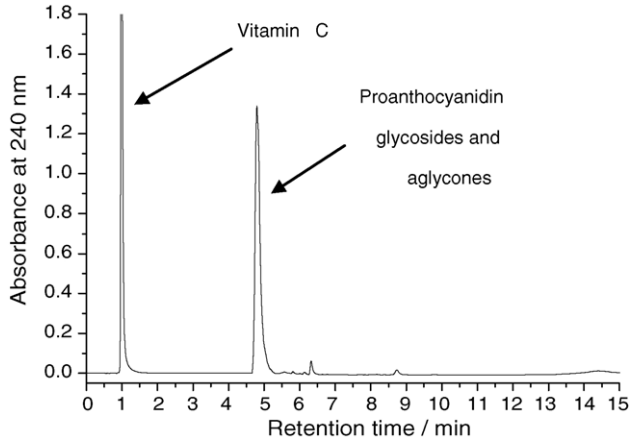

Fig. 6. HPLC traces of a 50% aq. ethanolic dog rose hip extract run with a Merck Superspher 100 RP-18 column (75 mm × 4 mm i.d., 3 μm) and a gradient consisting of 0.05 M H_3PO_4 (A) and MeCN (B): 0–2 min, 100% A (isocratic); 2–3.5 min, 0–70% B in A (linear gradient); 3.5–6 min, 70% B (isocratic); 6–8.5 min, 70–95% B in A (linear gradient); 8.5–9.5 min, 95–0% B in A (linear gradient); 9.5–15 min, 100% A (isocratic). Flow rate profile: 0–6 min, 1 mL min^{-1}; 6–6.5 min, 1–2 mL min^{-1} (linear gradient); 6.5–13.5 min, 2 mL min^{-1}; 13.5–14 min, 2–1 mL min^{-1} (linear gradient); 14–15 min, 1 mL min^{-1}.

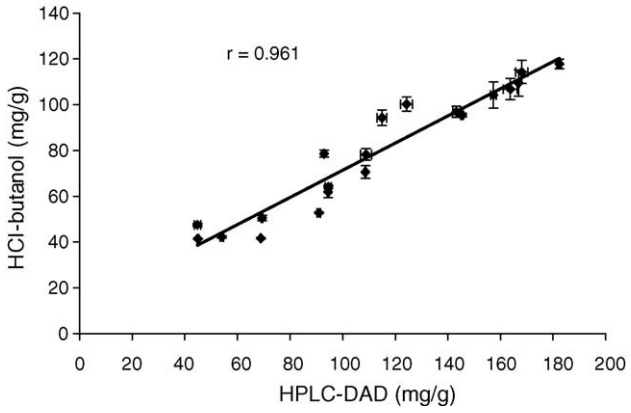

Fig. 7. Concentrations of proanthocyanidins (in mg/g dry weight) measured from twenty dog rose hip extracts with the developed HPLC–DAD and the conventional HCl–butanol assay. All extracts were measured in triplicate. Values show mean ± SE.

PAs by ESI. However, as the chromatographic hump of Fig. 1 was caused by tetra- to octameric PA glycosides (Fig. 5), it is apparent that these high-molecular-weight compounds are better detected with DAD than with ESI-MS. Therefore, to be able to reliably estimate at least the total concentration of PA glycosides and aglycones in a rose hip extract, we decided to develop a HPLC–DAD method for this purpose. Initially this was possible, since the whole PA glycoside hump (Fig. 1) was represented by compounds giving the UV spectra of PAs only. We simply accelerated the proportion of MeCN into 70% after the elution of Vitamin C, thus obtaining PAs as a single sharp peak (see Fig. 6). Moreover, the PA peak could be integrated accurately, in comparison to the hump shown in Fig. 1. To further demonstrate the PA-specificity of the developed method, we compared it with the spectrophotometric HCl–butanol assay that is presumably the most widely used method for the estimation of the total content of PAs from plants, mainly because of its simplicity and the absence of competing methods (but see, e.g., [16]). Fig. 7 shows a strong correlation between the results of the two methods and that the HCl–butanol assay tends to underestimate the content of rose hip PAs in comparison to HPLC–DAD (both methods were standardised by using the same PA fraction purified from rose hips). However, since quantifying the exact natural contents of secondary metabolites from plants presumably is almost an impossible task, even the results of the developed method cannot be considered as absolutely quantitative. Nevertheless, with HPLC–DAD the natural content of rose hip PAs is better estimated than with the HCl–butanol assay, of which PA recovery is much dependent of, e.g., the effectiveness of the chemical depolymerisation of PAs in the HCl–butanol mixture at 95 °C.

Although the HPLC–DAD method was developed especially for rose hip PAs, it allowed the simultaneous estimation of the content of Vitamin C as well. To avoid the depletion in the content of Vitamin C as a function of time, the extraction solvent (50% aq. EtOH) had to be adjusted to pH 2 by 0.05 M H_3PO_4 and the sample preparation simplified so that the extract was ready for HPLC analysis directly after its filtration, without the need for any time-taking concentration or fractionation steps (see Section 2 for details). This way Vitamin C remained stable for a minimum of 9 h after extraction. This enabled the extraction and analysis of 36 rose hip samples for the estimation of the total content of PA glycosides and aglycones, and Vitamin C during a total of 12 h only. Quantification limit for Vitamin C was 0.004 μg and for PAs 0.05 μg (calculated as PA equivalents by using a purified PA fraction of rose hips as a standard). For dog rose hips these limits were acceptable, since from the 275 hip samples so far analysed with the method, none contained Vitamin C or PAs less than 0.1 μg or 3.4 μg per injection, respectively, although the extraction was done from 10 mg hip samples only. It should be noted that this rapid method is suitable only for chemically well-characterised rose hips, such that are known to contain mainly PAs under the chromatographic hump. Some of the already analysed 275 dog rose hips contained enhanced levels of flavonoids (those reported in Table 2) that produced an almost shoulder-like peak

Fig. 5. Ion traces of proanthocyanidin (PA) glycosides emphasizing that glycosides of mono- to trimeric PAs (A–D) separate as nice peaks in reversed-phase HPLC, but from tetramers (E) towards heptamers (I) the separation is lost and compounds elute as a hump. (A): PA monomer monoglycoside, (B): PA dimer monoglycoside, (C): PA trimer monoglycoside, (D): PA trimer diglycoside, (E): PA tetramer triglycoside, (F): PA pentamer diglycoside, (G): PA pentamer triglycoside, (H): PA hexamer pentaglycoside, and (I): PA heptamer hexaglycoside.

just after the peak of PAs. Nevertheless, even with these kinds of "impure" samples the method is capable of estimating the content of PAs, given that the peak purity is first checked by the diode array detector.

4. Conclusions

On the basis of HPLC–ESI-MS analysis the abundance of individual PA aglycones and glycosides in rose hips was confirmed. While PA glycosides as such were found for the first time in rose hips, glycosides as large as tetra- to octamers were found, to our knowledge, only now in natural products on the whole. Already [17] noted—having found a single catechin glycoside from birch bark—that "the occurrence of the flavan-3-ol glycoside as a major metabolite in birch bark is significant in that such secondary products are rarely found in Nature in contrast to the ubiquitous flavonoid glycosides". We found PA glycosides as major phenolic metabolites in rose hips and flavonoid glycosides only in minor amounts. This unusual distribution of these compounds in rose hips enabled us to develop a rapid method for the estimation of the total concentrations of PA glycosides and aglycones in dog rose hip extracts. Simultaneously the Vitamin C (i.e., ascorbic acid) content of the hips can be screened as well, the total content of Vitamin C being determined after the reduction of dehydroascorbic acid (the oxidised, still active form of Vitamin C) into ascorbic acid. Moreover, while rose hips are an excellent source of well-known antioxidants like Vitamin C and carotenoids, also the PA aglycones found in hips of *R. canina* by [7] were shown to contribute to the total antioxidant activity of hip extracts. It is presumable that the PA glycosides found in this study could serve as efficient antioxidants as well, especially since [18] found mono- to trimeric PA glycosides of cacao liquor as good as or even stronger than α-tocopherol (Vitamin E) in both inhibiting the autooxidation of linoleic acid and in scavenging the DPPH radical. Clearly more studies are needed to examine the biological activities of the rose hip PA glycosides and the possibility to use dog rose hips in, e.g., functional food development. These studies will benefit from the developed HPLC–DAD assay; the reported HPLC-ESI-MS method could be used to verify the distribution of PA aglycones versus glycosides (Fig. 2) in the examined extracts.

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References

[1] S. Khanna, S. Roy, D. Bagchi, M. Bagchi, C.K. Sen, Free Radic. Biol. Med. 31 (2001) 38.
[2] S.B. Astley, Trends Food Sci. Technol. 14 (2003) 93.
[3] J.N. Losso, Trends Food Sci. Technol. 14 (2003) 455.
[4] H. Schroeder, H. Braun, Die Hagebutte, Ihre Geschichte. Biologie und ihre Bedeutung als Vitamin C-Träger, Stuttgart, WissenschaftlicheVerlagsgesellschaft, 1946.
[5] S. Stritzke, Die Hagebutte. Ein hochwertiger Vitaminspender, VEB Deutscher Landwirtschaftsverlag, Dresden, 1962.
[6] T. Hodisan, C. Socaciu, I. Ropan, G. Neamtu, J. Pharm. Biomed. Anal. 16 (1997) 521.
[7] D.A. Daels-Rakotoarison, B. Gressier, F. Trotin, C. Brunet, M. Luyckx, T. Dine, F. Bailleul, M. Cazin, J.-C. Cazin, Phytother. Res. 16 (2002) 157.
[8] E. Hvattum, Rapid Commun. Mass Spectrom. 16 (2002) 655.
[9] G. Werlemark, Acta Univ. Agric. Suec. 257 (2000) 1.
[10] E.-M. Choi, J.-K. Hwang, J. Ethnopharmacol. 89 (2003) 171.
[11] M. Karonen, J. Loponen, V. Ossipov, K. Pihlaja, Anal. Chim. Acta 522 (2004) 105.
[12] J.-P. Salminen, V. Ossipov, J. Loponen, E. Haukioja, K. Pihlaja, J. Chromatogr. A 864 (1999) 283.
[13] S. Ossipova, V. Ossipov, E. Haukioja, J. Loponen, K. Pihlaja, Phytochem. Anal. 12 (2001) 128.
[14] Y.-S. Bae, J.F. Burger, J.P. Steynberg, D. Ferreira, R.W. Hemingway, Phytochemistry 35 (1994) 473.
[15] J.-P. Salminen, V. Ossipov, E. Haukioja, K. Pihlaja, Phytochemistry 57 (2001) 15.
[16] G.E. Adamson, S.A. Lazarus, A.E. Mitchell, R.L. Prior, G. Cao, P.H. Jacobs, B.G. Kremers, J.F. Hammerstone, R.B. Bucker, K.A. Ritter, H.H. Schmitz, J. Agric. Food Chem. 47 (1999) 4184.
[17] H. Kolodziej, Phytochemistry 28 (1989) 3487.
[18] T. Hatano, H. Miyatake, M. Natsume, N. Osakabe, T. Takizawa, H. Ito, T. Yoshida, Phytochemistry 59 (2002) 749.